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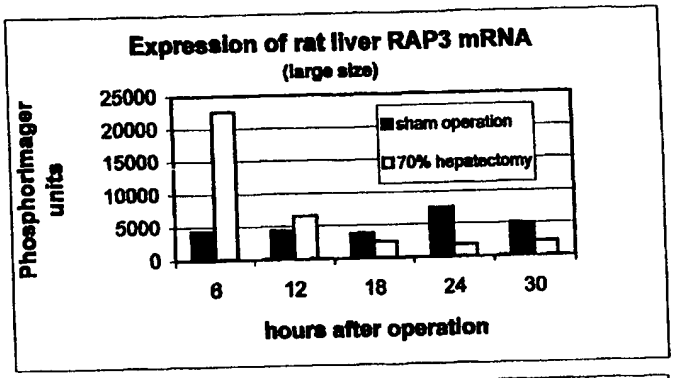
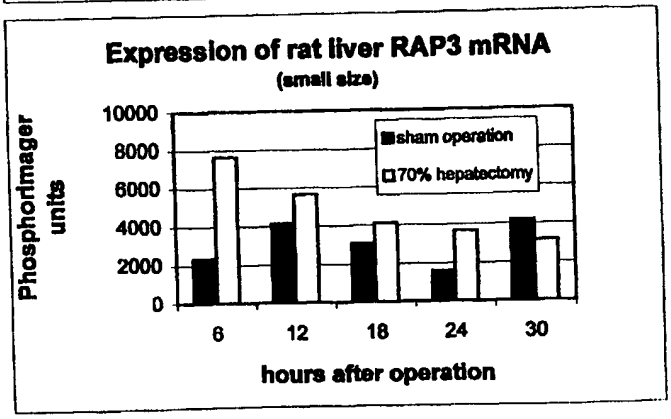
(54) Title: GENE AND PROTEIN INVOLVED IN LIVER REGENERATION

(57) Abstract

The present invention relates to a gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70 % homologous to the sequence of figure 1 or the sequence of figure 6, or the complementary strand thereof, for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence of figure 1 or the sequence of figure 6; protein encoded by said gene for use in diagnosis of liver regeneration and/or liver cell proliferation; and antibodies directed against this protein, a PCR primer comprising at least part of said gene as a probe, a single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from said gene as a probe an expression vector and a host cell comprising said nucleotide sequence, for use in a method for detecting the occurrence of liver cell proliferation in a subject.





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GENE AND PROTEIN INVOLVED IN LIVER REGENERATION

The present invention relates to the detection of a novel gene and protein involved in liver cell proliferation. The gene and protein and related molecules, such as nucleotide probes derived from the gene and antibodies directed to the protein form also part of the invention. The gene will be identified herein as RAP3 gene. The corresponding protein is called RAP3 protein.

The adult liver has the capacity to regenerate after damage or partial resection. This process may allow for recovery from hepatic injuries caused by viruses, toxins, ischemia, surgery, and auxiliary liver transplantation. Liver regeneration has been studied extensively in the rat after a 70% partial hepatectomy. During the first four hours following partial hepatectomy there is a rapid, transient transcriptional activation of genes involved in the immediate early response. After induction of these immediate early genes during the transition from the quiescent state of the liver (G_0) to the growth phase (G_1), a delayed early gene activation is initiated which peaks during the transition of the G_1 to the DNA synthesis phase (S phase).

In the research that led to the present invention novel genes involved in the delayed early response were identified by analyzing gene expression in rat liver at six hours after 70% partial hepatectomy. Upregulated genes were selected by cDNA subtractive hybridization. Upregulation was quantified by Northern blotting and the truly upregulated genes were characterized by sequence analysis.

Twelve genes were found to be upregulated at different degrees (1.5 to 10.4 fold) six hours after partial hepatectomy. Sequence analysis revealed that eight of the upregulated genes have previously been reported to be associated with liver regeneration or cell proliferation in general, one has previously been assigned an unrelated function and three have no sequence similarity to known genes.

The various upregulated genes showed two distinct gene expression patterns during a 30 hour period after partial hepatectomy. The first pattern has two peaks coincident with the G_1 phases of two consecutive hepatic cell cycles. The second one shows a narrow peak at six hours after which the gene is downregulated. The novel RAP3 gene (RAP: regeneration associated protein) which was most upregulated (3.3 fold), showed the latter gene expression pattern.

The full length cDNA of this gene was isolated from a rat liver cDNA library. Sequence analysis showed two full length cDNAs of 1282 and 1834 bp, respectively, encoding a novel protein of 367 amino acid residues. Figures 1A and 1B show the nucleotide sequence of the cDNA's. Figure 2 shows the derived amino acid sequence.

In addition, the cDNA of the human RAP3 gene was isolated. Sequence analysis showed twe cDNAs of 1282 and 1867 bp respectively, encoding a protein of 363 amino acids. Figures 6A and 6B show the nucleotide sequence of the cDNA's. Figure 7 shows the derived amino acid sequence. The 1867 human RAP3 cDNA shows a 76% homology with the 1834 rat RAP3 cDNA.

On the basis of this finding it became possible to design probes, primers and reagents for use in diagnosis. Probes and primers are generally based on the nucleotide sequence of the genes. Hybridization probes can comprise the whole or a large part of the coding or complementary strand of the sequence. PCR primers are typically smaller and encompass about between 10 and 50, preferably between 15 and 30, more preferably about 20 nucleotides.

The nucleotide sequences of some suitable PCR primers are given in the following table.

Table I

primer name	**nucleotide sequence**
F1RAP	5' GCA TCG TGG AAA GCA TGG CT 3'
F215RAP	5' GGG ACC CTT GAG AGA GCC TG 3'

F371RAP	5' CTT GAG GCA GCA GTT GAA AC 3'
F571RAP	5' TCC ACC CTT ATG CAG AAC GC 3'
F771RAP	5' AGT ACC TTC ATC CGT GTC AG 3'
F971RAP	5' CGC CTT CGC TCC AGA GTT GG 3'
F1171RAP	5' AGG GTG GAG GGT CCT GCA TA 3'
F1371RAP	5' GCA AGC CAG TAC TTG ACC GT 3'
F1621RAP	5' GTG GTC CTG CTG GGG GAT CA 3'
R234RAP	5' CAG GCT CTC TCA AGG GTC CC 3'
R420RAP	5' CTA CCT GCT CCA TCA GCT CG 3'
R570RAP	5' AGA GTT CTT TGA CTC GGT CC 3'
R770RAP	5' GAG CTC ATC TCG CAG CTG AT 3'
R970RAP	5' CTG TGG CTA GGC GGG GGT GG 3'
R1170RAP	5' CTG CCT ATT AGG CCA TGC TG 3'
R1370RAP	5' AGT CAG TCT CCC CCG CAC AC 3'
R1570RAP	5' TGG CAG GGA TGT ACA CAC TC 3'
R1837RAP	5' TTT CCA TCA TGA GCG TCT AT 3'

The hybridization probes can be labeled with a detectable label, such as a radioactive or biotin label.

Diagnosis of expression of the gene can be performed by means of a Northern blot. Total RNA or mRNA of a sample is separated on an agarose gel. The separation pattern is transferred to a nylon or nitrocellulose filter. An increase or decrease in the expression level is subsequently detected by hybridization with the above described hybridization probe. Typically a reference sample is included for comparison.

In case the protein is the basic macromolecule for diagnosis polyclonal or monoclonal antibodies are used for detection. The skilled person is very well capable of preparing such antibodies based on his common knowledge. Antibodies against the protein are part of the present invention.

Samples to be diagnosed can be a liver biopsy, plasma or serum. The latter can be used because the protein is secreted in the blood stream.

With the above described diagnostic methods an increase or decrease in the expression of the gene of the invention can be detected. The information that can thus be obtained is useful for establishing the efficacy of therapeutic agents stimulating liver regeneration and for patients who underwent an (auxiliary) liver transplantation and for monitoring patients treated with a bioartificial liver.

The invention is further illustrated in the following examples, which are in no way intended to be limiting to the invention. In the examples reference is made to the following figures:

Figure 1A is the nucleotide sequence of the 1282 bp cDNA.

Figure 1B is the nucleotide sequence of the 1834 bp cDNA.

Figure 2 shows the deduced amino acid sequence of the rap3 protein.

Figure 3 shows a polyacrylamide gel of liver cDNA fragments before and after subtraction. 26 cDNA fragments were found to be enriched after subtraction. Some of these are indicated by arrows. Lane 1 shows liver cDNA fragments of 6 hours 70% partial hepatectomy before subtraction. Lane 2 shows cDNA fragments of 6 hours 70% partial hepatectomy after subtraction.

Figure 4 shows the results of the Northern blot analysis of the temporal expression of RAP3 up to 30 hours after 70% partial hepatectomy. Panel A represents the Northern blot mRNA expression patterns at 3, 6, 12, 18, 24 and 30 hours after the 70% hepatectomy (hpx) and laparotomy (sham). Panel B represents the quantified hybridization signals indicated in PhosphorImager arbitrary units obtained at 6, 12, 18, 24 and 30 hours after the 70% hepatectomy and laparotomy.

The novel gene RAP3 is mostly upregulated 6 hours after partial hepatectomy after which it becomes downregulated.

Figure 5 shows a rat tissue Northern blot hybridized with a RAP3 cDNA probe. The RAP3 gene is specifically expressed in the liver.

Figure 6A shows the nucleotide sequence of the 1282 bp human RAP3 cDNA.

Figure 6B shows the nucleotide sequence of the 1867 bp human RAP3 cDNA (B).

Figure 7 shows the deduced amino acid sequence of the human rap3 protein.

Figure 8 shows the results of immunoblotting. About 6 ng of denatured rat RAP-3 protein was blotted on each strip. The strips were incubated with two antisera in various concentrations: 1-3: antiserum against purified native rat RAP3 protein (concentrations: 1:2000, 1:8000, 1:32000); 4-7: antiserum against purified denatured rat RAP3 protein (1:2000, 1:8000, 1:32000, 1:128000).

Figure 9 shows the results of an immunoblot after analyis with the program LumiAnalyst. 2.5 µl rat plasma was blotted per lane. The blot was incubated with antiserum against the denatured RAP3 protein. (hpx: plasma after 70% partial hepatectomy; sham: plasma after sham operation; positive control: about 10 ng purified denatured rat RAP3 protein).

EXAMPLES

EXAMPLE 1

Isolation of RAP3 gene associated with liver regeneration

1. Introduction

Recovery from Hepatic injuries caused by viruses, toxins, ischemia, surgery and auxiliary liver transplantation can be achieved by regeneration of the liver. The regeneration process has been studied extensively in the rat after a 70% partial hepatectomy.

During the first four hours following partial hepatectomy there is a rapid, transient transcriptional response. After this induction during the transition from

the quiescent state of the liver (G_0) to the growth phase (G_1), a delayed early gene activation is initiated, which peaks during the transition of the G_1 to the DNA synthesis phase (S phase).

This example demonstrates the isolation and identification of genes which are upregulated in the regenerating liver 6 hours after 70% partial hepatectomy.

2. Methods

2.1 Rat liver tissue preparation

Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were anesthetized with ether and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) (G.M. Higgins and R.M. Anderson, Arch. Pathol. **12**, 186 (1931)).For sham-operated animals, the liver was exposed by a midventral laparotomy.

The rats were allowed to recover from anesthesia. At 3, 6, 12, 18, 24, and 30 hours, respectively, after the 70% partial hepatectomy and sham surgery the animals were killed and the remaining liver was immediately harvested.

2.2 RNA isolation

Total liver RNA was isolated from liver tissue using the Trizol reagent kit (Life Technologies). Liver poly A^+ RNA was isolated from total liver RNA using oligo-(dT)-cellulose (Boehringer Mannheim GmbH) affinity chromatography as described previously (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: A laboratory Manual, Cold Spring Harbor, NY). To obtain highly pure poly A^+ RNA populations the oligo-(dT)-cellulose step was performed twice. The integrity of the poly A^+ RNA populations was determined on Northern blot by hybridization with glutathione-S transferase (data not shown).

2.3 PCR-select cDNA subtraction

The PCR-select cDNA subtraction kit (Clontech) was used to selectively amplify delayed early genes differentially expressed during liver regeneration. This method subtracts sequences common to both cDNA populations by suppressing undesirable PCR amplification, rather than by physically separating single stranded and double-stranded DNA. The 6 hours 70% partial hepatectomy liver poly A^+ population, containing the differentially expressed mRNA's, was compared with the 6 hours laparotomy liver mRNA population. Delayed-early genes start to appear 3 to 4 hours after the 70% partial hepatectomy. By using a laparotomy liver mRNA population rather than a normal liver mRNA population, the two populations were equalized for acute phase mRNA's, which are induced by the operation itself.

The PCR-select cDNA subtraction was performed according to the manufacturer's protocol with the following modifications. After two hybridizations, a nested PCR was used to selectively amplify the differentially expressed sequences. The second, nested PCR was performed in the presence of 0.5 μM [α-^{33}P]dATP (1200 Ci/mmol, final volume 25 μl). Subsequently, the amplified and differentially expressed cDNA fragments

were visualized on a denaturing 4% polyacrylamide DNA sequencing gel. An X-ray film (Biomax, Kodak) was exposed overnight to the unfixed, dried gel.

Figure 3 shows the results of the subtraction. Before subtraction (lane 1), the majority of the cDNA's were poorly identifiable, indicating the presence of many cDNA fragments of different molecular size. After subtraction (lane 2), 26 distinct cDNA fragments were observed as bands that were not apparent before subtraction.

2.4 Isolation and identification of visualized cDNA fragments

The 26 cDNA fragments that became visible after PCR-select cDNA subtraction were excised from the dried polyacrylamide gel and heated to 100°C for 5 minutes. Subsequently, 25 µl of the aqueous cDNA extract was used to amplify the cDNA by PCR with the nested primers used in the PCR-select cDNA subtraction. The PCR product was ligated into pCR II (Invitrogen), transformed into INVαF' competent cells, and plated out on agar plates containing ampicillin and X-Gal. Of each cloned PCR product, 6 white colonies were analyzed by PCR with T7 and SP6 primers for the presence of an insert.

Subsequently, plasmids containing an insert were purified using QIAprep (Qiagen) and the sequences of the inserts were determined using a dye terminator cycle sequencing system (Perkin Elmer) and a 377 DNA sequencer (ABI PRISM).

2.5 Northern blot analysis

To determine whether the expression of the genes found by the PCR-select subtractive hybridization is truly increased 6 hours after partial hepatectomy, Northern blot analysis was carried out using the purified cDNA fragments as probes. Poly A^+ RNA samples (0.8 µg) of the liver 6 hours after the hepatectomy and sham operation were electrophoresed on a 0.22 M formaldehyde-

1% agarose gel, and blotted onto a Hybond-N nylon membrane (Amersham) by capillary transfer overnight. For fixation of the poly A^+ RNA the blots were baked in an oven at 80°C for 2 hours.

The inserts of the sequenced clones were amplified by PCR using the nested primers of the PCR-select cDNA subtraction method. Qiaquick-spin columns (Qiagen) were used to purify the PCR products. The purified PCR products were radioactively labelled according to the hexamer-random primed method following the manufacturer's protocol (Promega), purified on Qiaquick-spin columns (Qiagen), and hybridized with the blots. Prehybridization (2 hours, 42°C) and hybridization (overnight, 42°C) was performed in 5 x SSPE, 50% formamide, 5 x Denhardt, 0.5% SDS, and 0.1 mg/ml sheared heat-denatured herring sperm DNA.

Following hybridization the blots were washed with 2 x SSC and 0.1% SDS for 15 min at room temperature and 42°C, respectively. Subsequently, the solution was replaced with 1 x SSC and 0.1% SDS and the blots were washed for 15 min at room temperature and at 42°C, respectively. The amount of hybridization was analyzed and quantified using a PhosphorImager (Molecular Dynamics).

The fold induction of the mRNA levels observed in the 70% partially hepatectomized animals over the sham operated animals after the specific hybridization was adjusted for variability in RNA loading.

The genes which were upregulated 1.5 times or more 6 hours after 70% hepatectomy together with their identity are given in Table II. Beside these twelve genes, three genes are indicated which expression could not be detected on Northern blot. The expression of the novel RAP3 gene was found to be upregulated 3.3 fold.

Table II

GENES UPREGULATED 6 HOURS AFTER A 70%		
Identity of gene	Function	Fold
Fibronectin	Liver regeneration	1.8
An intracisternal-A	Liver regeneration	1.8
γ-Actin	Liver regeneration	7
Ribophorin I	Liver regeneration	5.5, 1.7 & 2.3
α_2-Macroglobulin	Hepatocyte proliferation in vitro	5.4
Ribosomal Protein S5	Cell cycle	3.7 & 1.9
Ribosomal Protein L13	Cell cycle	2
Amyloid A Protein	Growth factor	10.4
Entactin		N.D.*
TCP-1-Containing Chaperonin related gene		1.5
31 kDa Putative Serine/ Threonine protein kinase		N.D.*
Novel RAP1**	Unknown	1.5
Novel RAP2**	Unknown	1.6
Novel RAP3**	Unknown	3.3
Novel RAP4**	Unknown	N.D.*

* N.D. = not detectable on Northern blot

** RAP1-3: Regeneration Associated Protein1-3)

EXAMPLE 2

Isolation and characterization of the full length RAP3 cDNA

Library screening and sequence analysis

A rat liver cDNA library was prepared from poly A^+ RNA isolated from the rat liver 6 hours after 70% hepatectomy. To obtain full length cDNA, the Great Lengths cDNA Synthesis Kit (Clontech) was used following the manufacturer's protocol. The adaptor ligated full

length cDNA inserts were cloned into the mammalian expression vector pCI at the EcoRI restriction site.

After transformation into DH10B electrocompetent cells (Gibco), the cDNA library was plated at a density of about 3,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with the ^{32}P-labeled RAP3 PCR fragment prepared according to the hexamer-random primed method following the manufacturer's protocol (Promega).

Following hybridization, the lift was washed and analyzed using a PhosphorImager (Molecular Dynamics). From the nine positive clones, the plasmid DNA was purified and the sequences of the inserts were determined using a Big dye terminator cycle sequencing system (Perkin Elmer) and an ABI PRISN 377 DNA sequencer (Perkin Elmer). The RAP3 cDNA was obtained by comparing the nine sequences with the sequence of the RAP3 PCR fragment. Two possible clones were detected and the start and end of the cDNA were confirmed by 5'- and 3'-RACE reactions carried out following the protocol of the Marathon cDNA Amplification kit (Clontech).

Based on the nucleotide sequence of the clones, PCR reactions were carried out with cDNA prepared from poly A$^+$ RNA of the rat liver 6 hours after 70% hepatectomy. The PCR products comprised the whole RAP3 cDNA, of which the nucleotide sequence was determined by bidirectionally sequencing the PCR products using 20 bp primers based on the already known nucleotide sequence data of the RAP3 cDNA.

Two RAP3 cDNA molecules were detected of 1282 and 1834 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 552 bp nucleotide part at the 3' side.

The nucleotide sequence of the 1282 bp RAP3 cDNA is as shown in Figure 1A.

The nucleotide sequence of the 1834 bp RAP3 cDNA is shown in Figure 1B.

Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the RAP3 protein. Its amino acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both RAP3 cDNA molecules encode the same RAP3 protein.

The amino acid sequence of RAP3 protein as deduced from the nucleotide sequence is shown in Figure 2.

EXAMPLE 3

Temporal expression between 3 and 30 hours after 70% partial hepatectomy

To define the temporal expression of the RAP3 gene, mRNA levels at 3, 6, 12, 18, 24, and 30 hours after the 70% partial hepatectomy and laparotomy were analyzed by the Northern blot procedure as described in example 1. Total RNA samples (20 µg) of the rat liver isolated at the various time points were electrophoresed. The Northern blot was hybridized with a radioactively labeled probe comprising basepairs 370 to 1834 of the large RAP3 cDNA. The result of the Northern blot and the quantified expression pattern are given in Figure 4. The expression pattern is presented as the hybridization signal in PhosphorImager arbitrary units obtained at 3, 6, 12, 18, 24, and 30 hours after the 70% partial hepatectomy and laparotomy.

Both RAP3 mRNA sizes are mostly upregulated 6 hours after partial hepatectomy after which they become downregulated.

The same procedure was carried out with probes of the other upregulated genes obtained by the PCR-select subtraction. Two distinct gene expression patterns during the 30 hour period after partial hepatectomy were found.

The first pattern has two peaks coincident with the G_1 phases of two consecutive hepatic cycles. The second one shows a narrow peak at six hours after which the gene is downregulated, just like the expression pattern of the novel RAP3 gene.

Determination of tissue specific expression

A Northern blot was prepared to determine expression of RAP3 mRNA in different tissues. The various tissues (skeletal muscle, spleen, liver, kidney, heart, lung and brain) were isolated from a female Wistar rat (175 g). The experiment was carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Total liver RNA was isolated from the tissues using the Trizol reagent kit (Life Technologies). A Northern blot was prepared from 20 µg total RNA samples and Northern blot analysis was carried out as described in example 1. A radioactively labeled probe comprising basepairs 370 to 1834 of the large RAP3 cDNA was used for the hybridization. The resulting Northern blot is given in Figure 5.

The RAP3 mRNA appeared to be clearly expressed in the liver and not at any detectable level in the other examined tissues. Because of this liver specificity and the 3.3 fold upregulation six hours after hepatectomy, the novel gene RAP3 was considered to be important in the process of liver regeneration.

EXAMPLE 4

Isolation and characterization of the full length human RAP3 cDNA

The Superscript Human liver cDNA library was purchased from Life Technologies. It contained liver cDNA from a 9-year old caucasian female directionally cloned into the pCMV'SPORT vector, and transformed into DH12S cells.

The cDNA library was plated at a density of about 25,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with a ^{32}P-labeled RAP3 cDNA fragment, consisting of the overlapping nucleotide sequence of the two rat RAP3 cDNA molecules. The labeling was carried out according to the hexamer random primed method following the manufacturer's protocol (Promega). Following hybridization, the membrane was washed with low stringency and analyzed using a PhosporImager (Molecular Dynamics).

Eight positive colonies and its near surroundings were cut from the agar plate and each transferred into 1 ml LB medium. 20 µl was plated on new agar plates and the hybridization protocol was repeated. Duplo clones of the eight positive colonies were isolated from the plate. The plasmid DNA was purified and the sequences of the inserts were determined using a Big Dye terminator cycle sequencing system (Perkin Elmer) and an ABI PRISM 377 DNA sequencer (Perkin Elmer). For sequencing initially the T7 and SP6 promotor primers were used and later on primers identical to twenty nucleotides of the sequenced part of the inserts.

By bidirectionally sequencing two human RAP3 cDNA nucleotide sequences were detected, both being presented by four of the eight examined clones. The two RAP3 cDNA molecules were 1322 and 1867 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 545 bp nucleotide part at the 3' side. The 1867 human RAP3 cDNA shows a 76% identity with the 1834 bp rat RAP3 cDNA.

The nucleotide sequence of the 1282 bp human RAP3 cDNA is shown in Figure 6A. The nucleotide sequence of the 1867 bp human RAP3 cDNA is shown in Figure 6B.

Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the human RAP3 protein. Its amino

acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both human rRAP3 cDNA molecules encode the same rap3 protein. The human RAP3 protein shows a 73% identity with the rat RAP3 protein, indicating that they indeed are the same protein but only expressed in different species.

The 363 residues counting amino acid sequence of the human RAP3 protein as deduced from the nucleotide sequence is shown in Figure 7.

EXAMPLE 5

Production of polyclonal antibodies against both rat and human RAP3

Expression of RAP3 protein

Both the rat and human cDNA sequences encoding the RAP3 protein without its presequence (the first 20 amino acid residues of the protein) were cloned into the pET-15b expression vector (Novagen) at the NdeI restriction site. The inserts were flanked by NdeI restriction sites, which had been introduced by PCR. By cloning at the NdeI site, a His-taq is expressed in front of the RAP3 protein, which is necessary for purification of the protein.

The vectors containing the RAP3 insert were transformed into the bacterial strain BL21(DE3) (Novagen). One colony was inocculated in 200 ml ampicillin containing LB medium. The medium was incubated at 37 ºC. After reaching an OD_{600} of 0.6/0.7 the medium was incubated for another four hours after which the cells were isolated.

The rat RAP3 protein was purified under non-denaturing conditions from the soluble fraction of the cells and under denaturing conditions from the insoluble fraction (inclusion bodies) of the cells. The human RAP3

protein was only purified under denaturing conditions from the insoluble fraction. The purification was carried out using His•Bind resin and columns (Novagen) following the manufacturer's protocol.

For identification the purified proteins were sequenced. Therefore the His-tag was removed from the proteins by cleavage with biotinylated thrombin (Novagen) following the manufacturer's protocol. The proteins were run on a 10% SDS-PAGE gel in Laemmli running buffer. The gel was blotted onto a PVDF membrane in CAPS buffer. The blot was shortly stained with Fast Blue (Pharmacia). The proteins were sequenced from blot by the Protein Research Facility Amsterdam of the E.C. Slater Institute in Amsterdam. The first thirteen amino acid residues of the expressed proteins after the His-tag were identified to be the same as the amino acid residues 21-33 of the rat and human RAP3 proteins as depicted in Figure 2 and 7 respectively. This indicates that the expected RAP3 proteins were expressed and can be used as antigens to raise polyclonal antibodies against the RAP3 proteins.

Immunisation

Rabbits were immunised with the various RAP3 proteins in order to obtain polyclonal antibodies against the rat and human RAP3 protein. About 200 µg purified RAP3 protein (including the His-tag) was suspended in Freund Complete Adjuvant and injected intracutaneously into 15 weeks old New Zealand White rabbits. After 2 months a booster of about 200 µg protein in Freund Incomplete Adjuvant was given intramuscular. One month later a booster of about 200 µg protein in PBS was given intramuscular and one to two weeks hereafter serum was collected from the rabbits.

The serum was tested in various concentrations for its ability to recognize the purified RAP3 protein. The sera against rat RAP3 were tested on the purified denatured rat RAP3 protein by immunoblotting. About 45 ng protein was run on a 10% SDS-PAGE gel in Laemmli running buffer.

The gel was blotted onto a PVDF membrane in Laemmli running buffer containing 20% methanol. The blot was cut into seven strips each loaded with approximately 6 ng rat RAP3 protein. The strips were blocked using Protifar (Nutricia), washed and incubated with antibodies (1 h, 20 ºC) according standard methods. The strips were incubated first with the sera against the rat RAP3 protein in various concentrations ranging from 1:2,000 to 1:128,000. Secondly the strips were incubated with alkaline phosphatase labeled goat anti-rabbit immunoglobulins (DAKO). The strips were now incubated with precipitating BM purple AP substrate (Boehringer Mannheim) following the manufacturer's protocol. Reaction of alkaline phospatase with the substrate leaves a dark purple band. The results are given in Figure 8. As shown a clear band at the right protein size appears even at the lowest concentrations. Serum from the rabbits before immunisations did not react with the RAP3 protein (data not shown). So polyclonal antibodies were obtained against the rat RAP3 protein. In the same way polyclonal antbodies against human RAP3 will be obtained.

EXAMPLE 6

Detection of RAP3 protein in rat plasma

Rat plasma isolation

Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were anesthesized with Hypnorm and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) {G.M. Higgins and R.M. Anderson, Arch. Pathol. **12**, 186 (1931)}. For sham-operated animals, the liver was exposed by a midventral laparotomy. The rats were allowed to recover from anesthesia. At 3, 6, 12 hours, respectively, after

the 70% partial hepatectomy and sham surgery the blood of the animals was heparinized and collected. The plasma of the rats was obtained by 5 minutes centrifugation at 1650g.

Immunoblotting

A Western blot was prepared as described before containing 2.5 µl plasma samples. Plasma samples of 3, 6 and 12 hours after both a sham operation and a 70% partial hepatectomy were examined. The blot was blocked (using Protifar), washed and incubated with antibodies (1 h, 20 ºC) according standard methods. The blot was incubated first with serum against the denatured rat RAP3 protein in a 1:6,000 dilution. Secondly the strips were incubated with horse radish peroxidase labeled goat anti-rabbit immunoglobulins (DAKO). The blot was now incubated with Lumi-light^{plus} Western blotting Substrate (Boehringer Mannheim) following the manufacturer's protocol. Analysis with the program LumiAnalyst gave the result as shown in Figure 9. RAP3 was found to be present in the plasma, so the liver excretes RAP3 into the blood. The appearing RAP3 has a somewhat smaller molecular weight than the positive control, because the positive control contains a His-tag in front of the protein. Especially the plasma samples of 6 and 12 hours after the hepatectomy showed an increase in RAP3 protein contents compared to the sham operation. So the concentration of the RAP3 protein is elevated in rat blood after 6 to 12 hours after a 70% hepatectomy.

EXAMPLE 7

Detection of changes of the amount of the RAP3 protein in the blood circulation

In order to detect changes in the amount of the RAP3 protein in the blood circulation a specific enzyme-linked immunosorbent assay (ELISA) is developed. Specific polyclonal and/or monoclonal antibodies are raised

against the whole protein or a part of the protein. The protein, human or rat, is expressed in a prokaryotic or eukaryotic expression system or part of the protein is synthesized chemically. Monoclonal and polyclonal antibodies, raised in rabbits, are isolated by common techniques as described previously (Coligan, J.E., Kruisbeek, A.M., Margulies, D.M., Shevach, E.M., and Strober, W. (1994) Current Protocols in Immunology, John Wiley & Sons, Inc. Chicester, New York).

CLAIMS

1. Gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70% homologous to the sequence shown in Fig. 1 or at least 70% homologous to the sequence shown Fig. 6, or the complementary strand thereof.

2. Gene as claimed in claim 1, **characterized in that** its cDNA has a nucleotide sequence which is at least 70% homologous to the nucleotide sequence as depicted in Fig. 1 or at least 70% homologous to the nucleotide sequence as depicted in Fig. 6, or the complementary strand thereof.

3. Gene as claimed in claims 1 or 2 for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence shown in Fig. 1 or with the gene sequence shown in Fig. 6.

4. Gene as claimed in claims 1 or 2 for use as a marker of liver proliferation.

5. Protein encoded by a gene as defined in claims 1 and 2 and comprising an amino acid sequence which is at least 70% homologous to the amino acid sequence given in Fig. 2 or at least 70% homologous to the amino acid sequence given in Fig. 7.

6. Protein as claimed in claim 5 having the amino acid sequence as depicted in Fig. 2 or Fig. 7.

7. Protein as claimed in claims 5 and 6 for use in diagnosis of liver regeneration and/or liver cell proliferation.

8. Antibodies directed against a protein as claimed in claims 5 and 6.

9. Antibodies as claimed in claim 7 for use in a method for detecting the occurrence of liver cell proliferation in a subject.

10. Antibodies as claimed in claim 8 or 9 which antibodies are monoclonal antibodies.

11. Antibodies as claimed in claim 8 or 9 which antibodies are polyclonal antibodies.

12. PCR primer, comprising at least part of the gene as claimed in claim 1.

13. PCR primer, comprising at least part of the nucleotide sequence as shown in Fig. 1 or at least part of the nucleotide sequence as shown in Fig. 6, or its complementary strand.

14. PCR primer as claimed in claims 12 and 13, wherein the "at least part of the nucleotide sequence" encompasses 10 to 50, preferably 15 to 30, more preferably about 20 nucleotides.

15. PCR primer as claimed in claims 12 to 14 having the nucleotide sequence as depicted in Table I or the complementary strand thereof.

16. PCR primer as claimed in claims 12 to 15 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

17. PCR primer as claimed in claims 12 to 15 for use in the detection of gene homologous to the gene as claimed in claims 1 to 3.

18. Single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from a gene as claimed in claims 1 to 3.

19. Single stranded nucleotide sequence as claimed in claim 18 which is antisense RNA.

20. Single stranded nucleotide sequence being at least in part complementary to the DNA or the cDNA from a gene as claimed in claims 1 to 3.

21. Single stranded nucleotide sequence as claimed in claims 18-20, further provided with a detectable label.

22. Nucleotide sequence as claimed in claims 18 to 21 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

23. Nucleotide sequence as claimed in claim 22, **characterized in that** the method in which the nucleotide sequence is used as a probe comprises the steps of:

a) obtaining a sample of a tissue or body fluid; and

b) detecting the amount of messenger RNA transcribed from a gene as claimed in claims 1 to 3 in that sample in comparison to a reference sample by means of the probe.

24. Nucleotide sequence as claimed in claim 23, wherein the sample is a liver biopsy, plasma or serum.

25. Nucleotide sequence as claimed in claim 18, 20 or 21 for use as a probe for screening a liver cDNA or genomic library.

26. Nucleotide vector comprising the nucleotide sequence as claimed in claims 18 to 25.

27. Expression vector comprising the nucleotide sequnece as claaimed in claims 18 to 25 in operative association with a regulatory element that controls expression of the nucleotide sequence in a host cell.

28. Host cell comprising the nucleotide sequence as claimed in claim 18-25.

29. Host cell comprising the nucleotide sequence as claimed in claim 18-25 in operative association with a regulatory element that controls expression of the nucleotide sequence in that host cell.

30. Transgenic animal in which the gene as claimed in claim 1 or 2 is an expresses transgene comprised in the genome of the animal.

31. Transgenic animal in which expression of the gene as claimed in claim 1 or 2 is prevented or repressed.

32. Method for treating liver disorder, comprising administering a compound that modulates the expression of the gene as claimed in claim 1 or 2, or the activity of the gene product of that gene to a patient in need of such treatment.

33. Method as claimed in claim 32 wherein the compound is an antisense or ribozyme molecule that blocks translation of the target gene.

34. Method as claimed in claim 32 wherein the compound is complementary to the 5' region of the target gene and blocks transcription via triple helix formation.

35. Method as claimed in claim 32 wherein the compound is an antibody that neutralizes the activity of the target gene product.

36. Method as claimed in claim 32 wherein the compound enhances the expression of the target gene, or the synthesis or activity of the gene product.

37. Method for treating liver disorder comprising administering nucleic acid encoding the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

38. Method for treating liver disorder comprising administering an effective amount of the gene product of the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

39. Method for enhancing the growth or regeneration of liver tissue comprising treating the liver tissue with an effective amount of the gene product of the gene as claimed in claim 1 or 2.

40. Method as claimed in claim 39 wherein the liver tissue is extracorporeal.

41. Method as claimed in claim 39 wherein the liver tissue is intracorporeal.

```
  1  GCATCGTGGA AAGCATGGCT GCCGTCATCA CCTGGGCACT CGCCCTCCTC
 51  TCAGTGTTTG CAACTGTACA GGCGAGGAAG AGCTTCTGGG AGTACTTCGG
101  CCAGAACAGC CAGGGCAAAG GCATGATGGG CCAGCAGCAG AAGCTGGCAC
151  AGGAGAGCCT GAAAGGTAGC TTGGAGCAAG ACCTCTACAA TATGAACAAT
201  TTCCTAGAAA AGCTGGGACC CTTGAGAGAG CCTGGGAAGG AGCCTCCTCG
251  GCTGGCACAG GATCCAGAAG GCATTCGGAA GCAGTTGCAG CAAGAGCTGG
301  AGGAAGTGAG CACACGCCTG GAGCCCTACA TGGCTGCAAA GCACCAGCAG
351  GTCGGCTGGA ACCTGGAGGG CTTGAGGCAG CAGTTGAAAC CCTACACGGT
401  CGAGCTGATG GAGCAGGTAG GCCTGAGCGT GCAGGATCTG CAAGAACAGC
451  TGCGCATGGT GGGAAAAGGC ACCAAGGCCC AGCTCCTGGG GGGCGTGGAT
501  GAGGCGATGA GCCTGCTGCA GGATATGCAA AGTCGAGTGC TGCACCATAC
551  GGACCGAGTC AAAGAACTCT TCCACCCTTA TGCAGAACGC TTGGTGACTG
601  GAATTGGGCA CCATGTGCAG GAGCTGCACC GGAGTGTTGC TCCTCACGCA
651  GTTGCCAGCC CCGCGAGACT CAGTCGCTGC GTGCAGACCC TGTCCCACAA
701  ACTCACACGT AAGGCGAAGG ACTTGCACAC CAGCATCCAA CGCAACCTGG
751  ATCAGCTGCG AGATGAGCTC AGTACCTTCA TCCGTGTCAG CACAGACGGG
801  GCAGACAACA GAGACTCCCT GGACCCTCAA GCTCTCTCTG ACGAGGTCCG
```

FIG. 1A-1

```
 851  CCAGAGACTC CAGGCTTTTC GACATGACAC CTACCTGCAG ATCGCTGCAT
 901  TCACTCAGGC CATTGACCAG GAGACCGAGG AAATCCAGCA CCAGCTGGCA
 951  CCACCCCCGC CTAGCCACAG CGCCTTCGCT CCAGAGTTGG GACACTCAGA
1001  CAGTAATAAG GCCCTGAGCA GACTGCAGAG CCGGCTGGAC GACCTCTGGG
1051  AAGATATTGC CTATGGCCTT CATGACCAGG GCCATAGTCA GAATAACCCT
1101  GAGGGTCACT CAGGTTAACT CTGCAGCTCG TTGTCTGGAC CCTGAGCCTT
1151  CAGCATGGCC TAATAGGCAG AGGGTGGAGG GTCCTGCATA CTATTGGCGA
1201  GGCCACCAAA GGTGCTGCTG CCCCAACCTG TCTGGCCTCC TCAACTCCCC
1251  CACTCAGGTG CATTACACTC AGTAGGTTTG GC
```

FIG. 1A-2

```
  1  GCATCGTGGA AAGCATGGCT GCCGTCATCA CCTGGGCACT CGCCCTCCTC
 51  TCAGTGTTTG CAACTGTACA GGCGAGGAAG AGCTTCTGGG AGTACTTCGG
101  CCAGAACAGC CAGGGCAAAG GCATGATGGG CCAGCAGCAG AAGCTGGCAC
151  AGGAGAGCCT GAAAGGTAGC TTGGAGCAAG ACCTCTACAA TATGAACAAT
201  TTCCTAGAAA AGCTGGGACC CTTGAGAGAG CCTGGGAAGG AGCCTCCTCG
251  GCTGGCACAG GATCCAGAAG GCATTCGGAA GCAGTTGCAG CAAGAGCTGG
301  AGGAAGTGAG CACACGCCTG GAGCCCTACA TGGCTGCAAA GCACCAGCAG
351  GTCGGCTGGA ACCTGGAGGG CTTGAGGCAG CAGTTGAAAC CCTACACGGT
401  CGAGCTGATG GAGCAGGTAG GCCTGAGCGT GCAGGATCTG CAAGAACAGC
451  TGCGCATGGT GGGAAAAGGC ACCAAGGCCC AGCTCCTGGG GGGCGTGGAT
501  GAGGCGATGA GCCTGCTGCA GGATATGCAA AGTCGAGTGC TGCACCATAC
551  GGACCGAGTC AAAGAACTCT TCCACCCTTA TGCAGAACGC TTGGTGACTG
601  GAATTGGGCA CCATGTGCAG GAGCTGCACC GGAGTGTTGC TCCTCACGCA
651  GTTGCCAGCC CCGCGAGACT CAGTCGCTGC GTGCAGACCC TGTCCCACAA
701  ACTCACACGT AAGGCGAAGG ACTTGCACAC CAGCATCCAA CGCAACCTGG
751  ATCAGCTGCG AGATGAGCTC AGTACCTTCA TCCGTGTCAG CACAGACGGG
801  GCAGACAACA GAGACTCCCT GGACCCTCAA GCTCTCTCTG ACGAGGTCCG
```

FIG. 1B-1

```
 851  CCAGAGACTC CAGGCTTTTC GACATGACAC CTACCTGCAG ATCGCTGCAT
 901  TCACTCAGGC CATTGACCAG GAGACCGAGG AAATCCAGCA CCAGCTGGCA
 951  CCACCCCCGC CTAGCCACAG CGCCTTCGCT CCAGAGTTGG GACACTCAGA
1001  CAGTAATAAG GCCCTGAGCA GACTGCAGAG CCGGCTGGAC GACCTCTGGG
1051  AAGATATTGC CTATGGCCTT CATGACCAGG GCCATAGTCA GAATAACCCT
1101  GAGGGTCACT CAGGTTAACT CTGCAGCTCG TTGTCTGGAC CCTGAGCCTT
1151  CAGCATGGCC TAATAGGCAG AGGGTGGAGG GTCCTGCATA CTATTGGCGA
1201  GGCCACCAAA GGTGCTGCTG CCCCAACCTG TCTGGCCTCC TCAACTCCCC
1251  CACTCAGGTG CATTACACTC AGTAGGTTTG GCAAACACAG CTTCCGGTGC
1301  TCATTTGGGA TCCTAAGGAG CAAGAGTGGG GTGAAGGGAG TGGGGAGATG
1351  GTGTGCGGGG GAGACTGACT GCAAGCCAGT ACTTGACCGT TGCTAGAAAC
1401  CTGTGTCACT ACAACCTGGA GCCCGGCTCC TATTACTTCA TGCCTGATGG
1451  TCGCTGTTAT AGTCGGTCTA CAGAGGGGAA CTCCTGTCTC CCCAGGGTTG
1501  TCATGACAGC CTTTGTTGGA AGAGAGCAGG AGAACATGAC ACGTATGATG
1551  GAGTGTGTAC ATCCCTGCCA GTGGTCCTGC TGGGGGAATC AGTGATGGGA
1601  TAAATGTGTG CATCCCTGCA GTGGTCCTGC TGGGGGATCA GTGATGGGAT
1651  GGGGCAGAGC CCCTATTTCC TTAGAGAACT CTAACCCAAA TAAGGAACTG
```

FIG. 1B-2

```
1701  AGCCCTCTGC AGTGAGGGCT TCTGAAAACC CTGTACATAG CAAACTGTGT
1751  GCCCTCTTCA TCATGCAGTC CCCACCTCCT GATTCTCGGG ATGGAACTGA
1801  CTTTTGGTTG GAATGAAATA GACGCTCATG ATGG
```

FIG. 1B-3


```
   1  MAAVITWALA LLSVFATVQA RKSFWEYFGQ NSQGKGMMGQ QQKLAQESLK
  51  GSLEQDLYNM NNFLEKLGPL REPGKEPPRL AQDPEGIRKQ LQQELEEVST
 101  RLEPYMAAKH QQVGWNLEGL RQQLKPYTVE LMEQVGLSVQ DLQEQLRMVG
 151  KGTKAQLLGG VDEAMSLLQD MQSRVLHHTD RVKELFHPYA ERLVTGIGHH
 201  VQELHRSVAP HAVASPARLS RCVQTLSHKL TRKAKDLHTS IQRNLDQLRD
 251  ELSTFIRVST DGADNRDSLD PQALSDEVRQ RLQAFRHDTY LQIAAFTQAI
 301  DQETEEIQHQ LAPPPPSHSA FAPELGHSDS NKALSRLQSR LDDLWEDIAY
 351  GLHDQGHSQN NPEGHSG*
```

FIG. 2

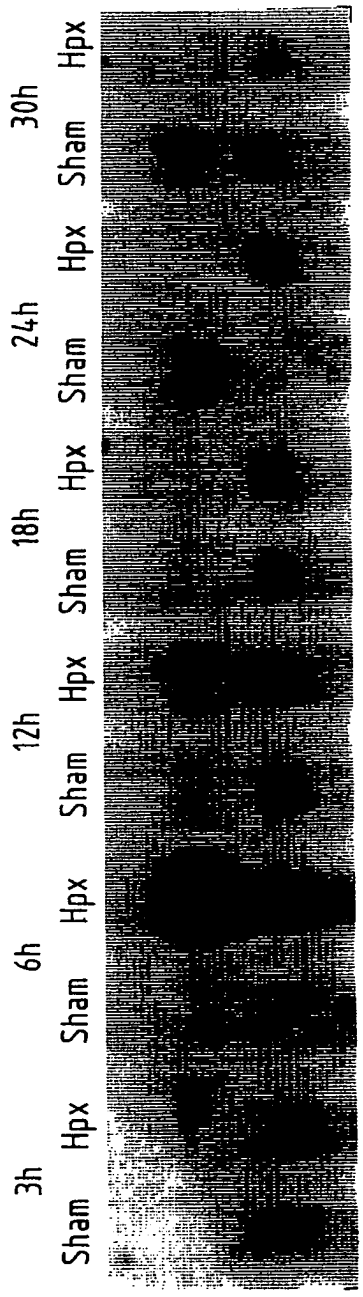


FIG. 4A

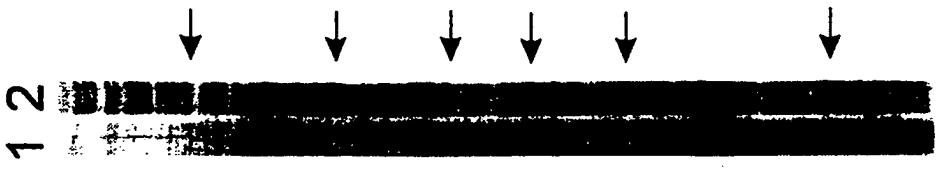
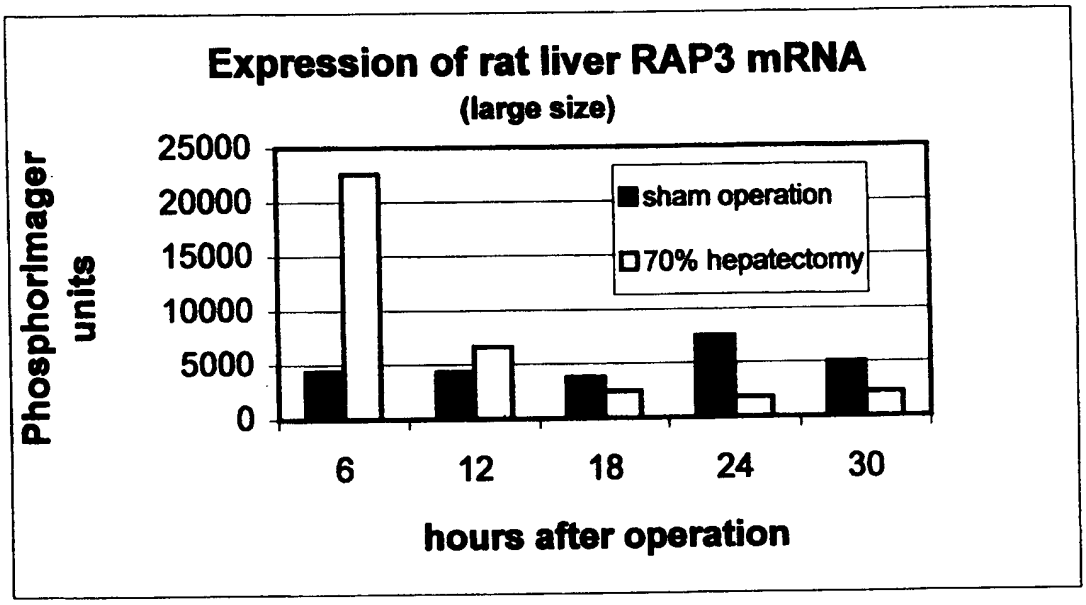


FIG. 3


Expression of rat liver RAP3 mRNA
(large size)
Phosphorimager units
25000
20000
15000
10000
5000
0
sham operation
70% hepatectomy
6
12
18
24
30
hours after operation

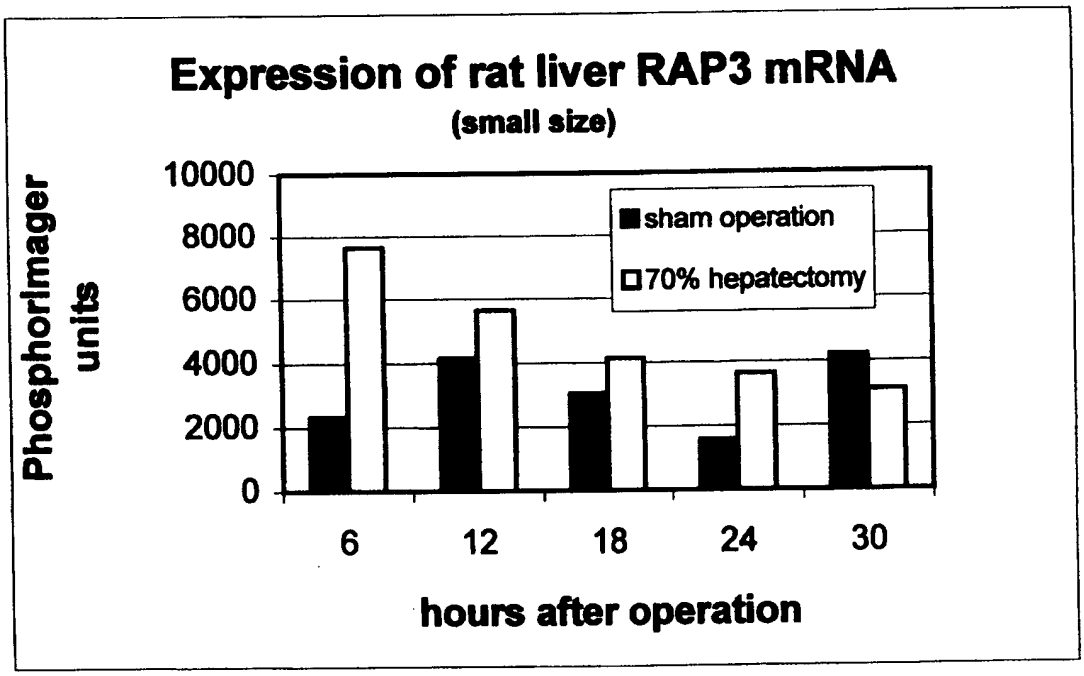

Expression of rat liver RAP3 mRNA
(small size)
Phosphorimager units
10000
8000
6000
4000
2000
0
sham operation
70% hepatectomy
6
12
18
24
30
hours after operation

FIG. 4B

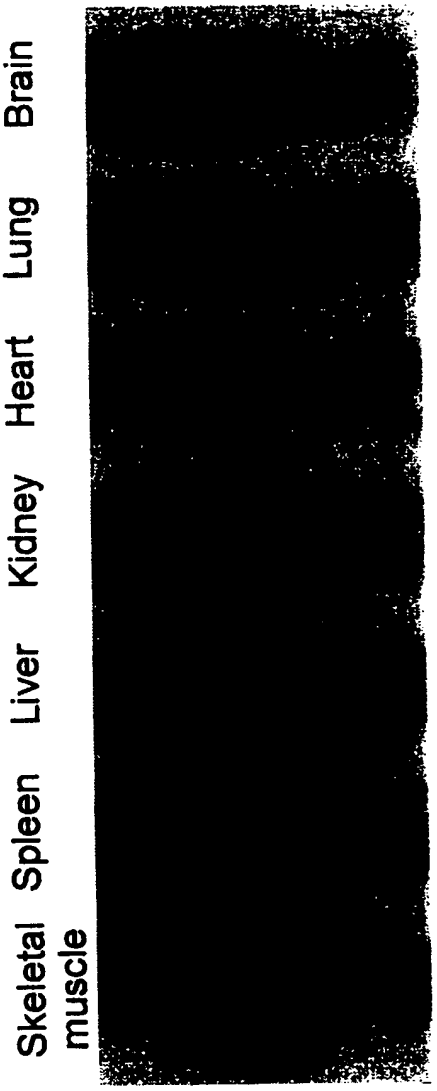


FIG. 5

```
  1  GAGCAGATAA TGGCAAGCAT GGCTGCCGTG CTCACCTGGG CTCTGGCTCT
 51  TCTTTCAGCG TTTTCGGCCA CCCAGGCACG GAAAGGCTTC TGGGACTACT
101  TCAGCCAGAC CAGCGGGGAC AAAGGCAGGG TGGAGCAGAT CCATCAGCAG
151  AAGATGGCTC GCGAGCCCGC GACCCTGAAA GACAGCCTTG AGCAAGACCT
201  CAACAATATG AACAAGTTCC TGGAAAAGCT GAGGCCTCTG AGTGGGAGCG
251  AGGCTCCTCG GCTCCCACAG GACCCGGTGG GCATGCGGCG GCAGCTGCAG
301  GAGGAGTTGG AGGAGGTGAA GGCTCGCCTC CAGCCCTACA TGGCAGAGGC
351  GCACGAGCTG GTGGGCTGGA ATTTGGAGGG CTTGCGGCAG CAACTGAAGC
401  CCTACACGAT GGATCTGATG GAGCAGGTGG CCCTGCGCGT GCAGGAGCTG
451  CAGGAGCAGT TGCGCGTGGT GGGGGGAAGAC ACCAAGGCCC AGTTGCTGGG
501  GGGCGTGGAC GAGGCTTGGG CTTTGCTGCA GGGACTGCAG AGCCGCGTGG
```

FIG. 6A-1

```
551  TGCACCACAC CGGCCGCTTC AAAGAGCTCT TCCACCCATA CGCCGAGAGC
601  CTGGTGAGCG GCATCGGGCG CCACGTGCAG GAGCTGCACC GCAGTGTGGC
651  TCCGCACGCC CCCGCCAGCC CCGCGCGCCT CAGTCGCTGC GTGCAGGTGC
701  TCTCCCGGAA GCTCACGCTC AAGGCCAAGG CCCTGCACGC ACGCATCCAG
751  CAGAACCTGG ACCAGCTGCG CGAAGAGCTC AGCAGAGCCT TTGCAGGCAC
801  TGGGACTGAG GAAGGGGCCG GCCCGGACCC CCAGATGCTC TCCGAGGAGG
851  TGCGCCAGCG ACTTCAGGCT TTCCGCCAGG ACACCTACCT GCAGATAGCT
901  GCCTTCACTC GCGCCATCGA CCAGGAGACT GAGGAGGTCC AGCAGCAGCT
951  GGCGCCACCT CCACCAGGCC ACAGTGCCTT CGCCCCAGAG TTTCAACAAA
1001 CAGACAGTGG CAAGGTTCTG AGCAAGCTGC AGGCCCGTCT GGATGACCTG
1051 TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGG
```

FIG. 6A-2

```
1101  GGACCCCTGA GGATCTACCT GCCCAGGCCC ATTCCCAGCT CCTTGTCTGG

1151  GGAGCCTTGG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCT

1201  GTTGGGTGGA GGGTGGAAGG TCCTGTGCAG GACAGGGAGG CCACCAAAGG

1251  GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG

1301  CATTACATTC ACCAGGCTTT GC
```

FIG. 6A-3

```
  1  GAGCAGATAA TGGCAAGCAT GGCTGCCGTG CTCACCTGGG CTCTGGCTCT
 51  TCTTTCAGCG TTTTCGGCCA CCCAGGCACG GAAAGGCTTC TGGGACTACT
101  TCAGCCAGAC CAGCGGGGAC AAAGGCAGGG TGGAGCAGAT CCATCAGCAG
151  AAGATGGCTC GCGAGCCCGC GACCCTGAAA GACAGCCTTG AGCAAGACCT
201  CAACAATATG AACAAGTTCC TGGAAAAGCT GAGGCCTCTG AGTGGGAGCG
251  AGGCTCCTCG GCTCCCACAG GACCCGGTGG GCATGCGGCG GCAGCTGCAG
301  GAGGAGTTGG AGGAGGTGAA GGCTCGCCTC CAGCCCTACA TGGCAGAGGC
351  GCACGAGCTG GTGGGCTGGA ATTTGGAGGG CTTGCGGCAG CAACTGAAGC
401  CCTACACGAT GGATCTGATG GAGCAGGTGG CCCTGCGCGT GCAGGAGCTG
451  CAGGAGCAGT TGCGCGTGGT GGGGGGAAGAC ACCAAGGCCC AGTTGCTGGG
501  GGGCGTGGAC GAGGCTTGGG CTTTGCTGCA GGGACTGCAG AGCCGCGTGG
```

FIG. 6B-1

```
 551  TGCACCACAC CGGCCGCTTC AAAGAGCTCT TCCACCCATA CGCCGAGAGC
 601  CTGGTGAGCG GCATCGGGCG CCACGTGCAG GAGCTGCACC GCAGTGTGGC
 651  TCCGCACGCC CCCGCCAGCC CCGCGCGCCT CAGTCGCTGC GTGCAGGTGC
 701  TCTCCCGGAA GCTCACGCTC AAGGCCAAGG CCCTGCACGC ACGCATCCAG
 751  CAGAACCTGG ACCAGCTGCG CGAAGAGCTC AGCAGAGCCT TTGCAGGCAC
 801  TGGGACTGAG GAAGGGGCCG GCCCGGACCC CCAGATGCTC TCCGAGGAGG
 851  TGCGCCAGCG ACTTCAGGCT TTCCGCCAGG ACACCTACCT GCAGATAGCT
 901  GCCTTCACTC GCGCCATCGA CCAGGAGACT GAGGAGGTCC AGCAGCAGCT
 951  GGCGCCACCT CCACCAGGCC ACAGTGCCTT CGCCCCAGAG TTTCAACAAA
1001  CAGACAGTGG CAAGGTTCTG AGCAAGCTGC AGGCCCGTCT GGATGACCTG
1051  TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGG
```

FIG. 6B-2

```
1101  GGACCCCTGA GGATCTACCT GCCCAGGCCC ATTCCCAGCT CCTTGTCTGG
1151  GGAGCCTTGG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCT
1201  GTTGGGTGGA GGGTGGAAGG TCCTGTGCAG GACAGGGAGG CCACCAAAGG
1251  GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG
1301  CATTACATTC ACCAGGCTTT GCAAACCCAG CCTCCCAGTG CTCATTTGGG
1351  AATGCTCATG AGTTACTCCA TTCAAGGGTG AGGGAGTAGG GAGGGAGAGG
1401  CACCATGCAT GTGGGTGATT ATCTGCAAGC CTGTTTGCCG TGATGCTGGA
1451  AGCCTGTGCC ACTACATCCT GGAGTTTGGC TCTAGTCACT TCTGGCTGCC
1501  TGGTGGCCAC TGCTACAGCT GGTCCACAGA GAGGAGCACT TGTCTCCCCA
1551  GGGCTGCCAT GGCAGCTATC AGGGGAATAG AAGGGAGAAA GAGAATATCA
1601  TGGGGAGAAC ATGTGATGGT GTGTGAATAT CCCTGCTGGC TCTGATGCTG
```

FIG. 6B-3

1651 GTGGGTACGA AAGGTGTGGG CTGTGATAGG AGAGGGCAGA GCCCATGTTT

1701 CCTGACATAG CTCTACACCT AAATAAGGGA CTGAACCCTC CCAACTGTGG

1751 GAGCTCCTTA AACCCTCTGG GGAGCATACT GTGTGCTCTC CCCATCTCCA

1801 GCCCCTCCCT CTGGGTTCCC AAGTTGAAGC CTAGACTTCT GGCTCAAATG

1851 AAATAGATGT TTATGAT

FIG. 6B-4

```
  1  MAAVLTWALA LLSAFSATQA RKGFWDYFSQ TSGDKGRVEQ IHQQKMAREP
 51  ATLKDSLEQD LNNMNKFLEK LRPLSGSEAP RLPQDPVGMR RQLQEELEEV
101  KARLQPYMAE AHELVGWNLE GLRQQLKPYT MDLMEQVALR VQELQEQLRV
151  VGEDTKAQLL GGVDEAWALL QGLQSRVVHH TGRFKELFHP YAESLVSGIG
201  RHVQELHRSV APHAPASPAR LSRCVQVLSR KLTLKAKALH ARIQQNLDQL
251  REELSRAFAG TGTEEGAGPD PQMLSEEVRQ RLQAFRQDTY LQIAAFTRAI
301  DQETEEVQQQ LAPPPPGHSA FAPEFQQTDS GKVLSKLQAR LDDLWEDITH
351  SLHDQGHSHL GDP*
```

FIG. 7

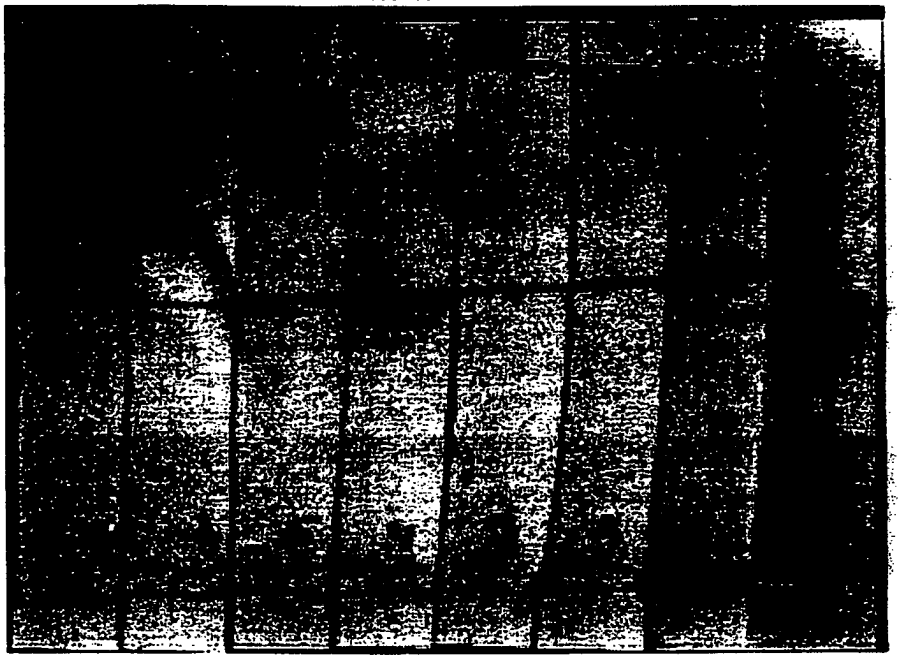


FIG. 8

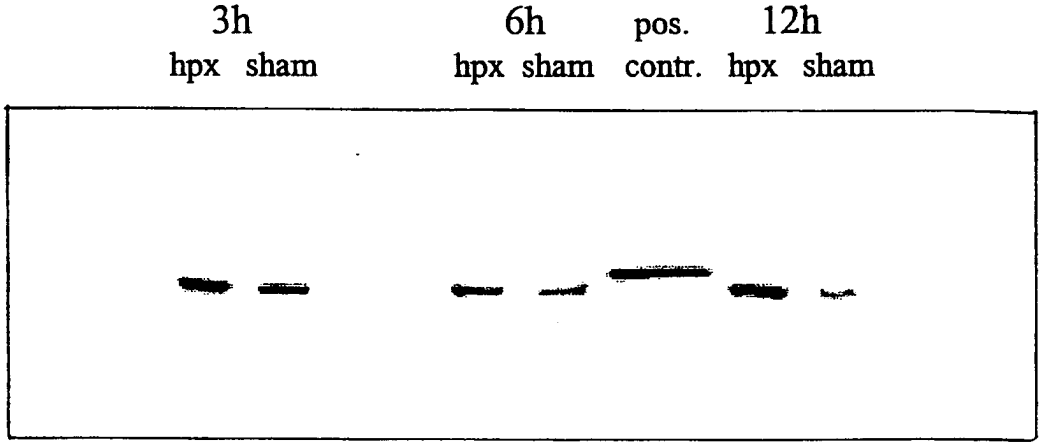


FIG. 9